Preparation for Sorting

General Information

Successful sorting depends on a variety of factors, including sample preparation, inclusion of controls, instrument set-up and sample tube or plate preparation.

Buffers for Sorting

basic PEB buffer: PBS (Ca/Mg^{++} free) supplemented with 2 mM EDTA and 0.5% BSA

If your cells tend to clump, you may consider making one of the following changes to your cell sorting buffer:

- Use calcium/magnesium free buffer!
- Add 0.5-2% BSA or FBS
- Add 10-25 mM HEPES, pH 7.0
- Add 2-5 mM EDTA
- if the cell preparation induces increased cell death
  - Add 25-50 µg/ml DNAse I + 5mM $MgCl_2$ (no EDTA then),
  - Add 1% Accutase

If medium is needed for better viability: Use the versions without phenol red, Ca2+ or Mg2+ and add 1 mM EDTA and 25 mM HEPES if your cells are sticky.

NOTE: High-glucose can not only lead to clogging of the Nozzle but also to **clogging of the Sample Line**, which is even worse because it cannot be cleaned it as well.

In-house Sample Preparation Protocol

1. Do not prepare the first sample before Startup and QC has passed (*it might take longer if there are problems with the sorter, so it makes no sense to trypsinize cells before you know everything is valid*)

2. Trypsinize your first dish of cells, stop trypsinization with growth medium and resuspend cells in a defined volume

TIPP: If you are using adherent cells, you should check the cells under a microscope before stopping the dissociation reaction to ensure that the majority of the cells are in a single cell suspension. Stopping the reaction too early can lead to large aggregates, resulting in poor sorting.

3. Determine the total cell number by counting the cells

4. Pellet your cells and resuspend them in 1 ml of PEB buffer per $2x10^6$ cells - example: if you have $8x10^6$ cells in your cell suspension, resuspend in 4 ml PEB buffer

TIPP: you might bring extra buffer. If the samples are too concentrated, extra buffer can be added at the sorter to optimize the event rate.

5. Always resuspend cells into a single cell suspension and remove large cell aggregates by filtering through a **40 µm or smaller cell strainer** before sorting (*only filter a maximum of 2 ml of cell suspension at a time, keep the rest of the cells on ice in case it is needed later. The reason for this is that processing the first 2 ml of cell suspension can take up to 2 hours depending on the sorting goal, transfection rate etc. and the cells will either die or start to clump after 2 hours; therefore, it is recommended to filter the second batch if needed*)

6. Transfer the cells into 5 ml FACS tubes *(Consider that you might want to keep your cells aseptic for further culture, e.g., bring your cells in solid cap tubes, filter cap tubes are open to the environment and can lead to contamination of your samples*)

7. Prepare 2 sterile FACS tubes with 1 ml of complete growth medium (*you only need one to start with if you are performing a one-way sort, the second is a backup that is only needed if the first becomes full and can be used for the next sort if it is not needed*)

TIPP: collection tubes can be pre-coated with whole serum, PBS with 20% FBS, or PBS with 5% BSA at room temperature for 2 hours, or at 4°C overnight to increase recovery.

During the Sort

1. Consider which temperature is best for your cells before, during and after sorting and keep your cells at the appropriate temperature for optimal health.

TIPP: Keeping your cells on ice will minimize clumping tendency.

2. Start with the required control sample, define sorting gate & strategy and start sorting

   a. A fully **unstained or mock transfected sample** is necessary to see the autofluorescence of your cells
   b. Whenever an experiment is run with more than one fluorochrome, **single color controls** must be recorded in order to calculate compensation to correct for fluorescent spillover (use the same fluorochrome used in the experiment and at least as bright as in the experimental samples)
   c. To determine appropriate gating when using more than two fluorochromes, the use of Fluorescence Minus One controls (**FMO controls**) is recommended
   d. Antibody-stained cells:
      i. When using fluorochrome conjugated secondary antibody, a **secondary antibody alone control** is recommended to determine any unspecific binding of the secondary antibody to the sample
      ii. **negative control** (fully stained sample known to be negative for your marker of interest to exclude cross-reactivity or false positive results)
      iii. **positive control** (fully stained sample known to be positive for your marker of interest to control experiment performance)

3. After completing sorting, note how many cells in total were sorted into your collection tube

4. Pellet your cells, resuspend them in growth medium and seed in an appropriate well plate or flask